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Multiple Cos2/Ci interactions regulate Ci subcellular localization through microtubule dependent and independent mechanisms

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Abstract

The Hedgehog (Hh) family of secreted proteins governs many developmental processes in both vertebrates and invertebrates. In *Drosophila*, Hh acts by blocking the formation of a truncated repressor form of Cubitus interruptus (Ci) and by stimulating the nuclear translocation and activity of full-length Ci (Ci155). In the absence of Hh, Ci155 is sequestered in the cytoplasm by forming protein complexes with Costal2 (Cos2), Fused (Fu) and Suppressor of Fused [Su(fu)]. How complex formation regulates Ci155 subcellular localization is not clear. We find that Cos2 interacts with two distinct domains of Ci155, an amino (N)-terminal domain (CDN) and a carboxyl (C)-terminal domain (CORD), and Cos2 competes with Su(fu) for binding to the N-terminal region of Ci155. We provide evidence that both N- and C-terminal Cos2 binding domains are involved in the cytoplasmic retention of Ci155 in imaginal discs. Treating imaginal discs with microtubule-destabilizing reagent nocodazole promotes nuclear translocation of Ci155, suggesting that the microtubule network plays an important role in the cytoplasmic retention of Ci155. In addition, we find that adding a nuclear localization signal (NLS) to exposed regions of Ci155 greatly facilitates its nuclear translocation, suggesting that the cytoplasmic retention of Ci155 may also depend on NLS masking.

Keywords: Hh; Cos2; Ci; Signaling; Nuclear translocation; NLS; Microtubule; Protein complex

Introduction

The Hedgehog (Hh) signaling pathway governs multiple cellular processes including cell proliferation, cell fate determination and cell movement in animal development (Ingham and McMahon, 2001). Moreover, malfunction of Hh signaling activity has been implicated in numerous human disorders including cancers (Villavicencio et al., 2000). Hh exerts its biological influence via a conserved, yet still poorly defined signal transduction pathway. The reception system for the Hh signal consists of two multiple-span transmembrane proteins, Patched (Ptc) and Smoothened (Smo). In the absence of Hh, Ptc acts substoichiometrically to inhibit Smo activity through poorly understood mechanisms that appear to regulate Smo stability, phosphorylation and subcellular localization (Alcedo et al., 2000; Denef et al., 2000; Taipale et al., 2002; Zhu et al., 2003). In the Hh receiving cells, Hh physically interacts with Ptc and alleviates its inhibition on Smo (Chen and Struhl, 1996; Marigo et al., 1996; Stone et al., 1996).

Hh signal transduction culminates in the activation of Cubitus interruptus (Ci), a member of Gli family of Zn finger transcription factors (Ingham and McMahon, 2001). In imaginal disc development, Ci plays dual roles that are performed by two distinct forms. In the absence of Hh, full-length Ci (Ci155) undergoes proteolytic processing to generate a truncated form (Ci75) that functions as a repressor to block the expression of Hh responsive genes such as *dpp* as well as *hh* itself (Aza-Blanc et al., 1997; Dominguez et al., 1996; Methot and Basler, 1999). Hh inhibits Ci processing to generate the repressor form, leading to the accumulation of Ci155 (Aza-Blanc et al., 1997; Chen et al., 1999a). In addition, high levels of Hh signaling activity stimulate the activity of accumulated Ci155, which acts as a transcriptional activator to turn on the expression of other

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Hh responsive genes including *ptc* and *engrailed* (*en*) (Alexandre et al., 1996; Methot and Basler, 1999).

Inhibition of Ci processing alone is not sufficient to activate Ci155 (Chen et al., 1999a; Methot and Basler, 1999; Wang et al., 1999). Several other mechanisms are employed to block the activity of Ci155. For example, PKA phosphorylation of Ci155 appears to inhibit its transcriptional activity in addition to promoting its proteolysis (Chen et al., 1999b; Price and Kalderon, 1999; Wang et al., 1999). In addition, stoichiometric interaction between Su(fu) and Ci155 prevents its transformation into a labile hyperactive form (Ohlmeyer and Kalderon, 1998). A more recent addition to the list of multiple layers of Ci regulation is the control of Ci155 subcellular localization by both nuclear import and export mechanisms (Chen et al., 1999a; Wang and Holmgren, 1999, 2000; Wang et al., 2000). The C-terminal region of Ci155 from aa 703 to aa 850 mediates robust nuclear export of Ci155 (Aza-Blanc et al., 1997; Chen et al., 1999a). Ci nuclear export is not regulated by Hh signaling activity; however, it is blocked by Leptomycin B (LMB), a drug that inhibits CRM1-mediated nuclear export (Chen et al., 1999a; Kudo et al., 1998). In A-compartment cells distant from the A/P compartment boundary, Ci155 is sequestered in the cytoplasm, a process that requires Cos2, Fu and Su(fu) (Lefers et al., 2001; Methot and Basler, 2000; Wang and Holmgren, 2000; Wang et al., 2000). In A-compartment cells near the A/P compartment boundary, Hh stimulates nuclear translocation of Ci155 in a manner depending on Fu kinase activity (Wang and Holmgren, 2000; Wang et al., 2000).

Ci forms a large protein complex that includes Cos2 and Fu (Robbins et al., 1997; Sisson et al., 1997). In addition, the N-terminal region of Ci interacts with Su(fu) (Methot and Basler, 2000; Monnier et al., 1998). Deletion of this region from Ci promotes its nuclear translocation, suggesting that Su(fu) impedes Ci nuclear import through direct binding (Methot and Basler, 2000; Wang et al., 2000). Cos2 binds a 125 amino acid domain (CORD) in the C-terminal half of Ci that mediates its cytoplasmic retention by Cos2 (Wang et al., 2000). These observations support the view that complex formation among Ci, Cos2, Fu and Su(fu) regulates Ci nuclear translocation; however, the underlying mechanism is still unknown.

Here we provide evidence that cytoplasmic retention of Ci depends on at least two distinct mechanisms: microtubule tethering and NLS masking. In addition, we show that Cos2 interacts with Ci through two distinct domains in Ci, both of which are involved in cytoplasmic retention of Ci.

Materials and methods

Transgenes

All Ci deletion mutants were tagged with a double HA tag at their N termini and cloned into the pUAST vector (Brand and Perrimon, 1993; Wang et al., 1999). Ci76 has a C-terminal truncation at aa 703. Ci76ΔCDN has an internal deletion that removes aa 346–440 from Ci76. CiΔCORD has an internal deletion from aa 941 to aa 1065 in otherwise full-length Ci. CiΔCDN is a full-length Ci with an internal deletion that removes aa 346–440. CiΔNΔC contains double deletions that remove both aa 346–440 and aa 941–1065. To create Ci^{NLS}, an NLS from the SV40 large T antigen was introduced between the HA tag and the N-terminus of full-length Ci (for $Ci^{NLS\text{-}N}$), or before the stop codon (for $Ci^{NLS\text{-}C}$). A Flag-tag was fused to the N-terminus of Cos2 to generate Flag-Cos2, or to the C termini of Su(fu) and Fu to generate Flag-Su(fu) and Flag-Fu. The Flag-tagged Cos2, Su(fu) and Fu were then cloned into the pUAST vector. Gal4 driver lines used are *actin5C > CD2 > Gal4* (Pignoni et al., 1997) and *MS1096* (Capdevila and Guerrero, 1994).

Immunostaining and drug treatment of imaginal discs

Standard protocols were used for immunostaining of the third instar larval imaginal discs (Jiang and Struhl, 1998). Primary antibodies were monoclonal rat anti-Ci 2A1(Motzny and Holmgren, 1995), mouse anti-Arm (Peifer et al., 1994), mouse and rabbit anti-HA (Santa Cruz) and rabbit polyclonal anti-Flag (ABR). Secondary antibodies were from the Jackson Labs. Drug treatment of imaginal discs was performed as described (Wang et al., 2000). The imaginal discs were cultured in M3 medium containing 20–100 ng/ml LMB and/or 40 μg/ml Nocodazole for 3 h before immunostaining.

Cell culture, immunoprecipitation and Western blot

S2 cells were cultured in Schneider cell medium. DNA constructs expressing UAS transgenes and Ub-Gal4 were transiently transfected into S2 cells using the calcium-phosphate precipitation method. Proteins from cell lysates were immunoprecipitated with a mouse anti-HA antibody (Santa Cruz) as previously described (Robbins et al., 1997). The immunoprecipitates and aliquots of total lysates were analyzed by immunoblotting with either a mouse anti-Flag M2 (Sigma) or a rabbit anti-Su(fu) antibody (Stegman et al., 2000), and visualized by ECL Plus system (Amersham).

Yeast two-hybrid assay

For interaction between Cos2 and the N-terminal region of Ci, full-length Cos2 was fused to the LexA DNA-binding domain in the pEG202 vector, and various Ci fragments were fused to the Gal4 activation domain in the pACT2 vector. The LexA–Gal4 hybrid system was also used to map Cos2 domains that interactions with the N- or C-terminal region of Ci as well as to determine the interaction between Fu and Ci. To map the Cos2 domain that binds Fu, various Cos2 fragments were fused to the Gal4 DNA-

binding domain, transformed into yeast with a pACT2-Fu fusion construct. Filter assay and liquid β-galactosidase assays are as described (Durfee et al., 1993; Tall et al., 1999).

In vitro binding assay

The coding sequence for Ci76 (aa 1–703) was fused in frame with the glutathione S-transferase (GST) gene in pGEX-4T1. Recombinant GST-Ci76 fusion protein and GST were purified using Glutathione Sepharose 4B beads (Amersham Pharmacia) from bacterial extracts. The beads were incubated with ^{35}S-labeled Su(fu) or/and Cos2 proteins at 4°C for 1 h. The incubation mixtures were washed three times with cold PBS buffer containing 1% Triton X-100 and the precipitates were subjected to SDS-PAGE. ^{35}S-labeled proteins were visualized by exposing the gel on Kodak X-ray film. ^{35}S-labeled Su(fu) and Cos2 proteins were synthesized by using the TNT Coupled Reticulocyte Lysate System (Promega).

Results

Identification of a second Cos2 interacting domain in the N-terminal region of Ci

We have previously identified a 125 amino acid (aa) domain named CORD (aa 941–1065) in the C-terminal part of Ci that mediates Cos2 binding (Wang et al., 2000). The previous structure function study was carried out using Ci deletion mutants that lack the region N-terminus to the Zn-finger DNA binding domain. In this context, CORD is both necessary and sufficient for Cos2-mediated cytoplasmic retention (Wang et al., 2000). To determine if CORD is essential for cytoplasmic retention of full-length Ci, we deleted CORD from full-length Ci to generate CiΔCORD (Fig. 1). *UAS-HA-CiΔCORD* was expressed either alone or in conjunction with *UAS-Flag-Cos2* using the *actin5C > CD2 > Gal4* driver line. To allow detection of Ci after nuclear translocation, the imaginal discs were treated with LMB to block Ci nuclear export. This approach was used throughout this work where the nuclear translocation of full-length Ci or its derivatives were assessed. As shown in Fig. 2, HA-CiΔCORD is localized largely in the nucleus of wing disc cells after LMB treatment (Fig. 2A); however, it is retained in the cytoplasm when Flag-Cos2 is coexpressed (Fig. 2A′). More surprisingly, a Ci deletion mutant, Ci76, which corresponds to the repressor form of Ci (Aza-Blanc et al., 1997), was retained in the cytoplasm when it was coexpressed with Flag-Cos2 (Figs. 2B,B′). These observations suggest that the N-terminal region of Ci contains a Cos2 responsive element.

The N-terminal region of Ci (aa 1–346) binds Su(fu) and mediates Su(fu)-dependent cytoplasmic retention (Methot and Basler, 2000; Monnier et al., 1998; Wang et al., 2000). Since Cos2 can interact with Su(fu) indirectly through Fu (Monnier et al., 1998), cytoplasmic retention of

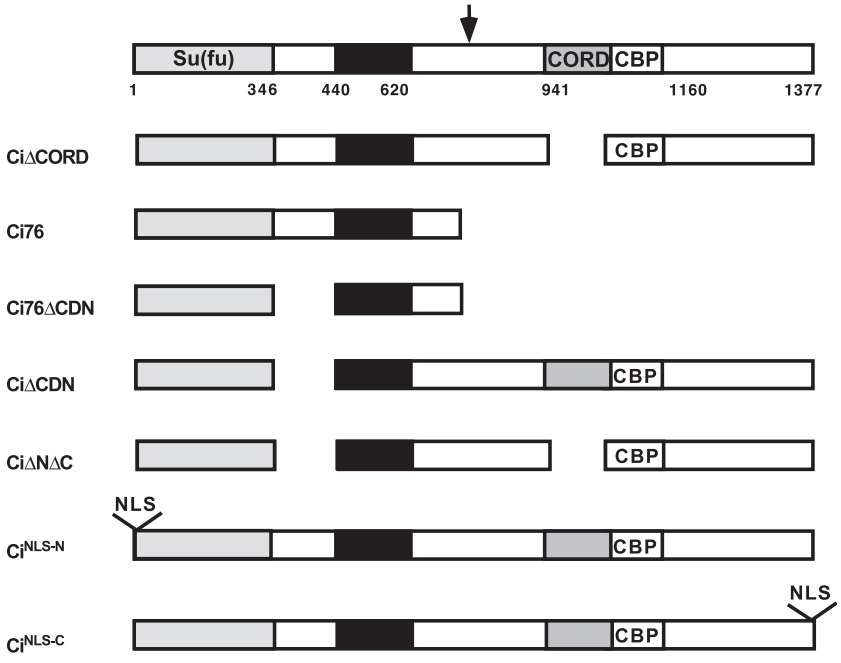

Fig. 1. A summary of Ci constructs used in this study. The top panel shows a schematic drawing of full-length Ci. Su(fu): Su(fu)-binding domain; ZF: zinc finger DNA binding domain; CBP: dCBP-binding domain; CORD: Cos2-responsive domain. All Ci constructs have two copies of HA-tag at their N-termini (not shown). CiΔCORD: full-length Ci with an internal deletion that removes CORD (from aa 942 to aa 1065). Ci76 contains Ci sequence from aa 1 to aa 703. Ci76ΔCDN: Ci76 with an internal deletion that removes CDN (from aa 346 to aa 440). CiΔCDN: full-length Ci with CDN deleted. CiΔNΔC: full-length Ci with both CDN and CORD deleted. Ci^{NLS-N} and Ci^{NLS-C} are full-length Ci with an NLS added to its N- and C-terminus, respectively.

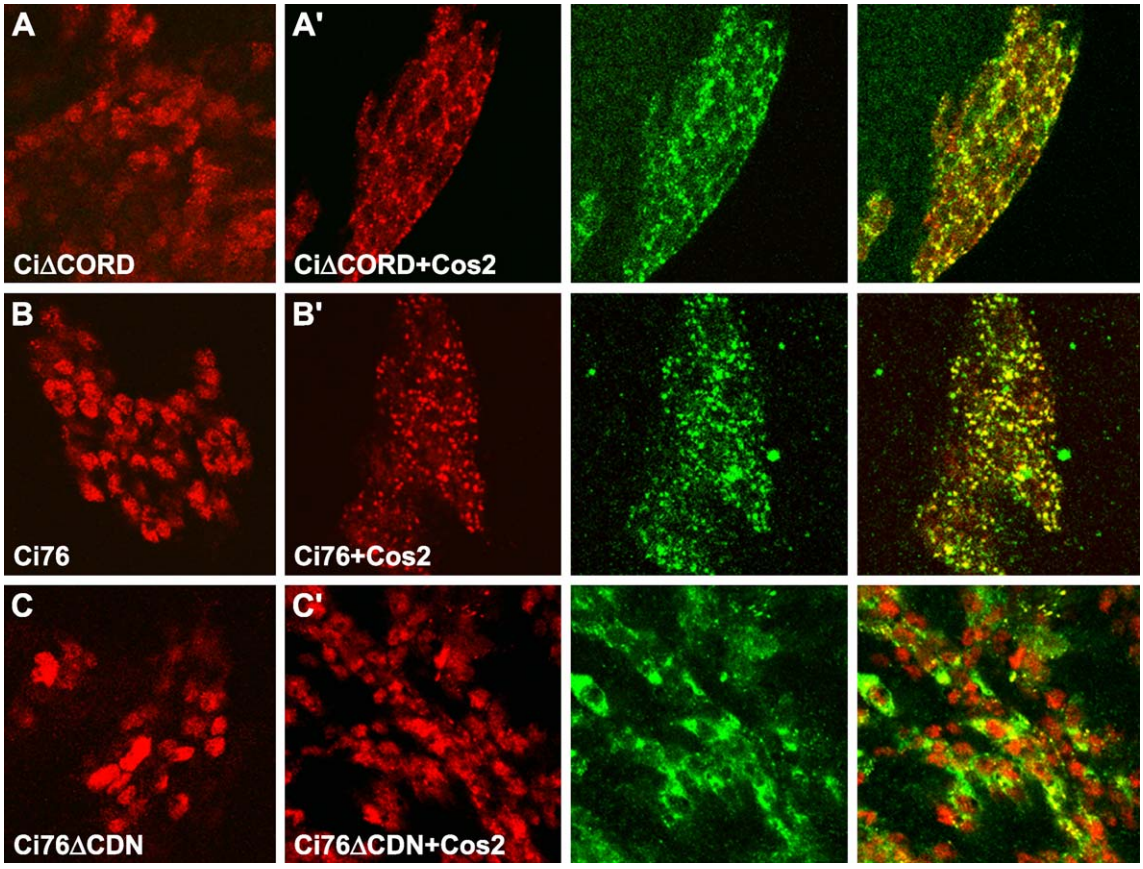

Fig. 2. Cos2 sequesters Ci through an N-terminal domain of Ci. HA-tagged CiΔCORD (A, A′), Ci76 (B, B′), and Ci76ΔCDN (C, C′) were expressed either alone (A–C) or in conjunction with a Flag-tagged Cos2 (A′ –C′) using the *act > CD2 > Gal4* driver line. The same *UAS-Flag-Cos2* line was used throughout this work. Wing discs expressing CiΔCORD (A, A′) were treated with 100 ng/ml LMB before immunostaining with anti-HA (red) and anti-Flag (green) antibodies. In the absence of Flag-Cos2, Ci deletion mutants are predominantly localized in the nucleus (A–C). When Flag-Cos2 is coexpressed, both CiΔCORD and Ci76 are sequestered in the cytoplasm (A′ , B′), whereas little if any Ci76ΔCDN is retained by Flag-Cos2 (C′).

CiΔCORD or Ci76 by Cos2 could be mediated by Su(fu). Alternatively, Cos2 could sequester Ci by directly binding to its N-terminal region. To distinguish these two possibilities, we carried out both yeast two-hybrid and coimmunoprecipitation assays to examine if Cos2 interacts with the N-terminal region of Ci. Various N-terminal fragments as well as CORD were fused to the Gal4 activation domain (GAD) to generate Ci-GAD fusion proteins (Fig. 3A). Ci-GAD constructs were transformed into yeast with a construct expressing the full-length Cos2 fused in frame with the LexA DNA binding domain, followed by a liquid assay (Tall et al., 1999). Ci fragment from aa 1 to aa 440 interacts with Cos2 in yeast, albeit with an affinity much lower than that of CORD/Cos2 interaction (Fig. 3B). Deletion from either end (Ci 212–440 or Ci 1–346) greatly reduces but not completely abolishes Cos2 binding, suggesting that the N-terminal Ci sequence required for Cos2 binding is dispersed. To further characterize the interaction between Cos2 and the N-terminal region of Ci, we deleted the region from aa 346 to aa 440 in Ci76 to generate Ci76ΔCDN (Fig. 1). HA-tagged Ci76 or Ci76ΔCDN were transfected into S2 cells with either Flag-tagged Cos2 or Flag-tagged Su(fu). Cell extracts were immunoprecipitated with an anti-HA antibody, followed by Western blot analysis with either anti-Flag or anti-Su(fu) antibody. As shown in Fig. 3C, Flag-Cos2 was coprecipitated with HA-Ci76 but not with HA-Ci76ΔCDN whereas equal amounts of Flag-Su(fu) were coprecipitated with both HA-Ci76 and HA-CiΔCDN. Hence, deletion of the Ci sequence from aa 346 to aa 440 compromises Cos2 binding to the N-terminal region of Ci, but does not significantly perturb Ci/Su(fu) interaction.

If Cos2 sequesters CiΔCORD or Ci76 by direct binding, one would predict that deletion of aa 346 to aa 440, which compromises Cos2 binding, should also affect Cos2-mediated cytoplasmic retention. To test this, *UAS-HA-Ci76* and *UAS-HA-Ci76ΔCDN* were expressed either alone or with *UAS-Flag-Cos2* in wing discs using the *actin5C > CD2 > Gal4* driver line. In contrast to HACi76, which is sequestered efficiently by Flag-Cos2 (Figs. 2B,B′), HA-Ci76ΔCDN is barely retained by Flag-Cos2 (Figs. 2C,C′). Taken together, these observations suggest that Cos2 directly binds the N-terminal region of Ci from aa 346 to aa 440, which we named CDN (Cos2 Responsive Domain in the N-terminal region of Ci), and CDN can mediate cytoplasmic retention by Cos2.

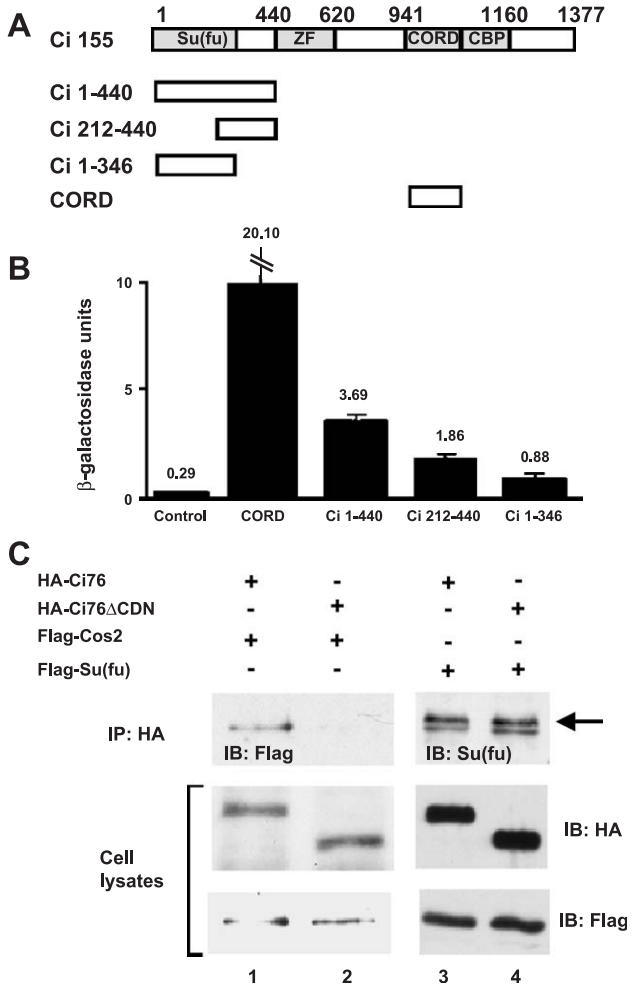

Fig. 3. Cos2 binds the N-terminal region of Ci. (A, B) Various N-terminal fragments of Ci and CORD were fused in frame with the Gal4 activation domain. The resulting Ci/Gal4 fusion constructs were transformed into yeast with a Cos2/LexA DNA binding domain fusion construct. Interactions were determined by a liquid assay. The relative strength of protein–protein interaction is reflected by the β-galactosidase activity (B). (C) S2 cells were transfected with HA-Ci76 (lanes 1 and 3) or HA-Ci76ΔCDN (lanes 2 and 4) with Flag-Cos2 (lanes 1 and 2) or Flag-Su(fu) (lanes 3 and 4). Cell lysates were immunoprecipitated (IP) with a mouse anti-HA antibody, followed by immunoblotting (IB) with a mouse anti-Flag or a rabbit anti-Su(fu) antibody. An aliquot of cell lysates, which corresponds to 20% of the material used in IP, was immunoblotted with an anti-HA (top) or anti-Flag (bottom) antibody. Ci76 but not Ci76ΔCDN pulled down Cos2 (lanes 1 and 2; top panel) whereas both pulled down equal amounts of Su(fu) (lanes 3 and 4; indicated by the arrow). Of note, the bottom band recognized by the anti-Su(fu) antibody is nonspecific.

Both CDN and CORD are involved in cytoplasmic retention of Ci

To determine the relative contribution of CDN and CORD in mediating cytoplasmic retention of full-length Ci by Cos2, we generated additional Ci deletion mutants that lack either CDN alone (CiΔCDN) or CDN and CORD simultaneously (CiΔNΔC) (Fig. 1). HA-tagged CiΔCDN or CiΔNΔC was expressed either alone or in conjunction with Flag-Cos2 in wing discs using the *Act > CD2 > Gal4* driver line. The wing discs were treated with 100 ng/ml LMB, followed by immunostaining with anti-HA and anti-Flag antibodies. As shown in Fig. 4, both HA-CiΔCDN and HA-CiΔNΔC are localized largely in the nucleus when expressed alone (Figs. 4A,B). Like HA-CiΔCORD, the majority of HA-CiΔCDN is retained in the cytoplasm when Flag-Cos2 is coexpressed (Fig. 4A′). By contrast, the majority of HA-CiΔNΔC is localized in the nucleus even when Flag-Cos2 is coexpressed (Fig. 4B′). Hence, both CDN and CORD can mediate cytoplasmic retention of Ci by overexpressed Cos2.

We then examined the effect of deleting CDN or CORD on cytoplasmic retention of Ci by endogenous Cos2. HA-Ci, HA-CiΔCORD, HA-CiΔCDN, and HA-CiΔNΔC were expressed in wing discs using the *MS1096* Gal4 driver line, which expresses Gal4 uniformly in the wing pouch regions (Wang et al., 1999), and their subcellular localization were examined by immunostaining after wing discs were treated with 50 ng/ml LMB. Of note, the *MS1096* Gal4 driver line is weaker than the *Act > CD2 > Gal4* driver line so that Ci transgenes were expressed at lower levels. In P-compartment cells, all forms of Ci are localized in the nucleus (data not shown). In A-compartment cells, however, the majority of HA-Ci is in the cytoplasm whereas HA-CiΔCORD appears to be evenly partitioned in the cytoplasm and nucleus (Figs. 4C,D). HA-CiΔCDN is largely in the nucleus whereas HA-CiΔNΔC appears to be completely in the nucleus (Figs. 4E,F). Thus, both CDN and CORD are required for efficient cytoplasmic retention of Ci by the endogenous Cos2, with CDN more potent than CORD.

Protein–protein interactions among Ci, Cos2 and Fu

CDN and CORD do not share apparent sequence similarity; thus, they may interact with different regions of Cos2. To map Cos2 domains that interact with CDN and CORD, various Cos2 fragments were fused to GAD and tested for binding to Ci-LexA DNA binding domain fusion proteins containing either CiN (aa 1–620) or CiC (aa 941–1377). Cos2 fragment from aa 143 to aa 647 (Cos2 143–647) binds both CiN and CiC; however, further deletion from either end (Cos2 205–750 or Cos2 143–547) diminished binding to CiN (Fig. 5A). These observations suggest that the Cos2 sequence involved in CDN binding is dispersed. By contrast, the Cos2 sequence that mediates CORD interaction can be narrowed down to two small discrete regions as Cos2 fragments both from aa 143 to aa 343 (Cos2 143–343) and from aa 343 to aa 447 (Cos2 343–447) interact with CiC (Fig. 5A).

Cos2 and Fu form a tight complex both in *Drosophila* early embryos and in cultured cells (Robbins et al., 1997; Sisson et al., 1997), suggesting that these two proteins may interact directly. To test this hypothesis and to define the region of Cos2 that mediates Fu interaction, we fused various Cos2 fragments to the LexA DNA binding domain and tested for interaction with a Fu-GAD fusion protein. As

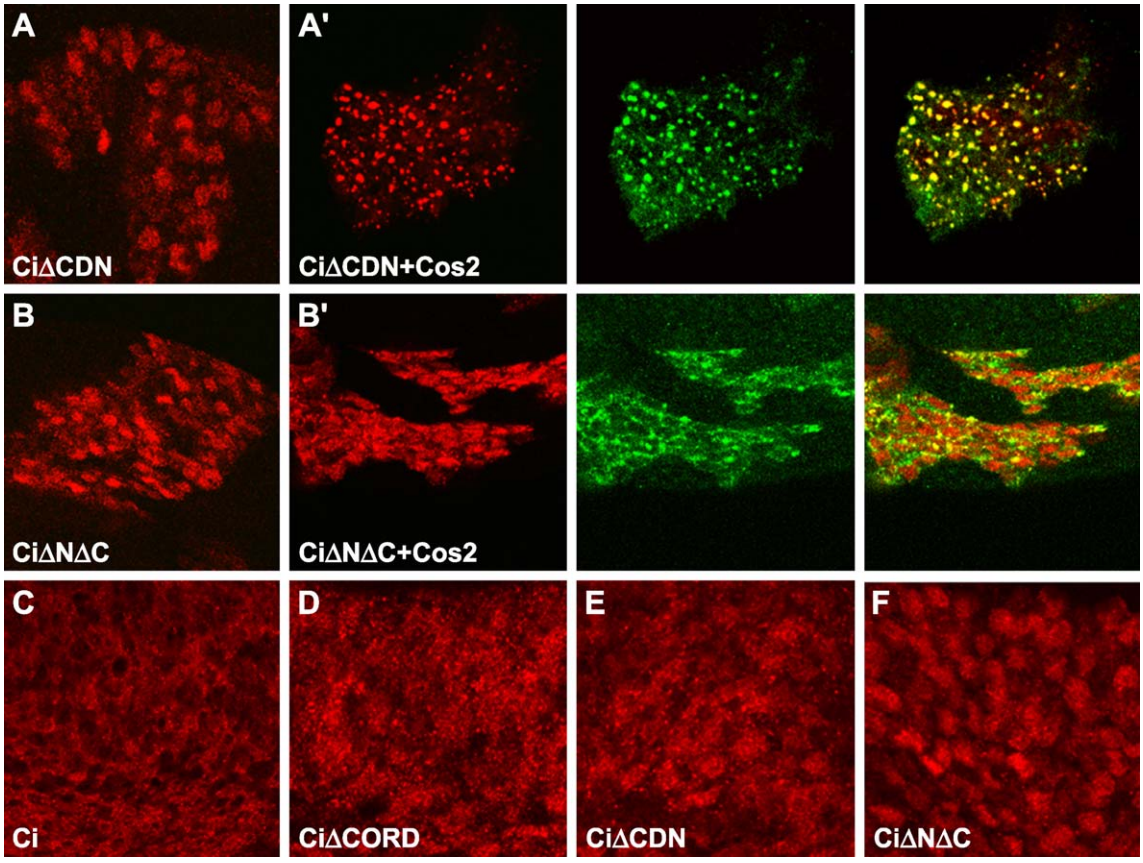

Fig. 4. Both CDN and CORD are involved in cytoplasmic retention of Ci. (A–B) HA-tagged CiΔCDN (A, A′) or CiΔNΔC (B, B′) was expressed either alone (A, B) or in conjunction with a Flag-tagged Cos2 (A′ , B′) using the *act > CD2 > Gal4* driver line. Wing discs were treated with 100 ng/ml LMB before immunostaining with anti-HA (red) and anti-Flag (green) antibodies. In the absence of Flag-Cos2, CiΔCDN and CiΔNΔC are predominantly localized in the nucleus (A, B). When Flag-Cos2 is coexpressed, CiΔCDN are sequestered in the cytoplasm (A′). In contrast, little if any CiΔNΔC is retained by Flag-Cos2 (B′). (C–F) HA-Ci (C), HA-CiΔCORD (D), HA-CiΔCDN (E), and HA-CiΔNΔC (F) were expressed in wing discs using *MS1096* driver line. Wing discs were treated with 50 ng/ml LMB, followed by immunostaining with an anti-HA antibody. High magnification view of A-compartment cells is shown in each panel.

shown in Fig. 5B, a Cos2 fragment containing sequence from aa 547 to aa 750 (Cos2 547–750) is sufficient to bind Fu. By contrast, Cos2 642–1245, which contains Cos2 sequence from aa 642 to aa 1245, fails to interact with Fu. These observations suggest that the central region of Cos2 from aa 547 to aa 642 is essential for Fu binding.

To determine whether Fu could also bind Ci directly, we tested whether LexA–CiN or LexA–CiC interacts with the Fu-GAD fusion protein in yeast. As shown in Fig. 5C, CiC but not CiN binds Fu, raising the possibility that Fu may directly interact with the C-terminal region of Ci in the Cos2/Ci/Fu ternary complex.

Cos2 and Su(fu) compete for binding to the N-terminal region of Ci

As both Cos2 and Su(fu) interact with the N-terminal region of Ci, this raises a possibility that Cos2 may compete with Su(fu) for binding to the N-terminal region of Ci. To test this possibility, we carried out competition experiments. The N-terminal half of Ci (Ci76) was fused in frame with the glutathione S-transferase (GST). Recombinant GST-Ci76 fusion proteins were purified using Glutathione Sepharose 4B beads from bacterial extracts, and were incubated with in vitro translated ^{35}S-labeled Su(fu) or Cos2 without or with increasing amounts of the corresponding competing protein. As shown in Fig. 6A, less Su(fu) was pulled down by GST-Ci76 in the presence of competing Cos2. Reciprocally, increasing amounts of Su(fu) decreased the amounts of Cos2 bound to GST-Ci76 (Fig. 6B). These results suggest that Su(fu) and Cos2 compete for binding the N-terminal region of Ci.

We next asked if Cos2 competes with Su(fu) for binding the N-terminal region of Ci in vivo. To do this, we examined if binding of Su(fu) to HA-CiΔCORD can be competed away by increasing Cos2 as deletion of CORD should bias Cos2 to bind the N-terminal region of Ci. Accordingly, HA-CiΔCORD and Flag-Su(fu) were cotransfected into S2 cells with or without Flag-Cos2. Cell extracts were immunoprecipitated with an anti-HA antibody, followed by Western blot analysis with either anti-Su(fu) or anti-Flag antibody.

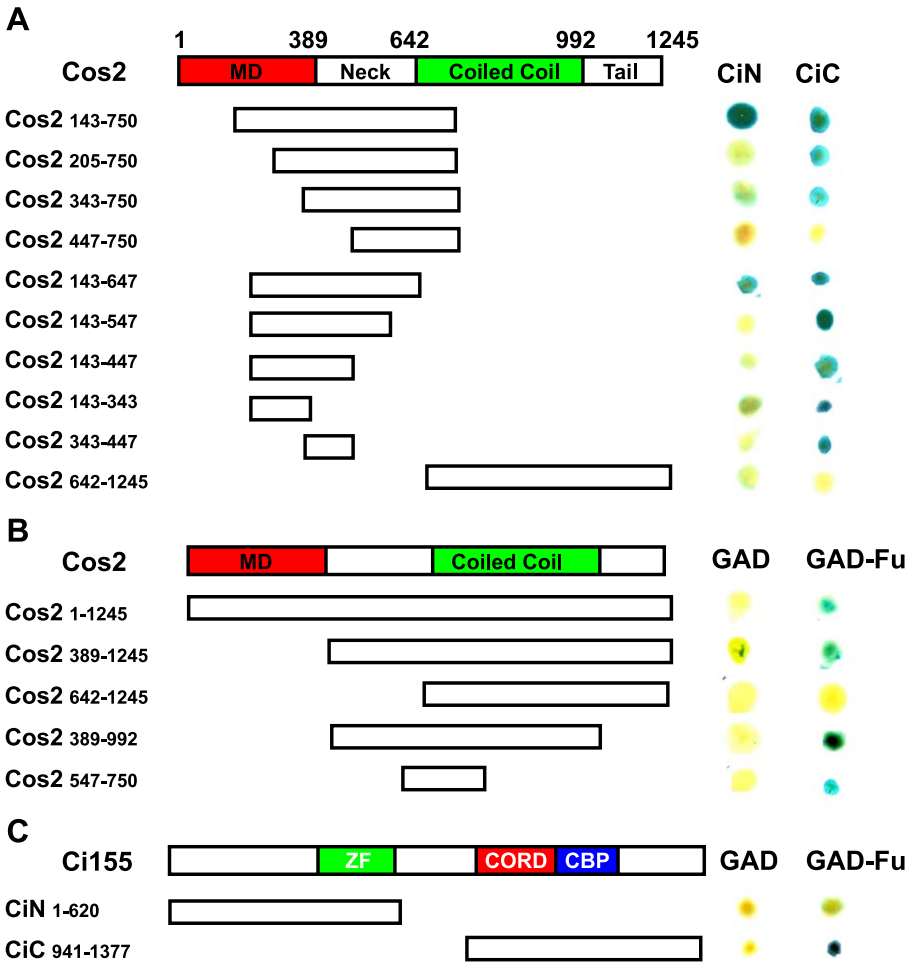

Fig. 5. Protein–protein interactions among Cos2, Fu and Ci. (A) A schematic drawing of Cos2 protein is shown at the top. MD: microtubule binding domain; Coiled-coil: 36 heptad repeats that are predicted to form coiled-coil. Constructs containing Cos2 fragments fused to the Gal4 activation domain were transformed into yeast with LexA DNA binding domain fusion constructs containing either CiN (from aa 1 to aa 620) or CiC (from aa 941 to aa 1396). (B) Full-length or Cos2 fragments were fused to the Gal4 DNA binding domain and transformed into yeast with full-length Fu fused to the Gal4 activation domain (GAD-Fu). The vector (GAD) was used as a control. (C) Ci fragments fused to the LexA DNA bind domain were transformed into yeast with either GAD-Fu or GAD. Protein–protein interactions were analyzed by a filter assay.

As shown in Fig. 6C, Su(fu) was coimmunoprecipitated with CiΔCORD in the absence of Flag-Cos2 (lane1); however, a diminishing amount of Su(fu) was coprecipitated with CiΔCORD when Flag-Cos2 was coexpressed (lane 2), suggesting that binding of Cos2 to CiΔCORD interferes with Su(fu)/CiΔCORD interaction. It appears that Cos2 competes with Su(fu) in binding Ci better in vivo than in vitro. One likely explanation is that the interaction between Cos2 and CND could be stabilized in vivo by Fu that bridges Cos2 and Ci.

In contrast to the interaction between Su(fu) and CiΔCORD, which is blocked by Cos2, binding of Su(fu) to CiΔCDN is not inhibited by increasing the amount of Cos2 (Fig. 6D). As a matter of fact, more Su(fu) was pulled down by CiΔCDN when Cos2 was coexpressed, suggesting that binding of Cos2 to the C-terminal region of Ci may promote Su(fu) to bind the N-terminal region of Ci, thus favoring the formation of a tetrameric complex containing Ci, Cos2, Fu and Su(fu) (Stegman et al., 2000).

Disruption of microtubules promotes Ci nuclear translocation

As Cos2, Fu and Ci cosediment with microtubules in a manner regulated by Hh, it has been proposed that Ci is sequestered in the cytoplasm through microtubule tethering (Robbins et al., 1997; Sisson et al., 1997). To test whether the microtubule network is essential for cytoplasmic retention of Ci, we examined the effect of disrupting microtubules on the subcellular localization of Ci. We perturbed microtubules by treating imaginal discs with a microtubule destabilizing drug, Nocodazole (Dong et al., 2000). To facilitate the detection of Ci155, we generated *slimb*$^{-}$ clones that accumulate Ci155 to high levels in wing discs (Jiang and Struhl, 1998). Imaginal discs carrying *slimb*$^{-}$ clones were incubated in M3 medium containing 50 ng/ml LMB with or without 40 μg/ml Nocodazole, followed by immunostaining with anti-Ci (2A1) and anti-Arm antibodies. As shown in Fig. 7, and consistent with our previous study (Wang et al., 2000), Ci155 accumulated

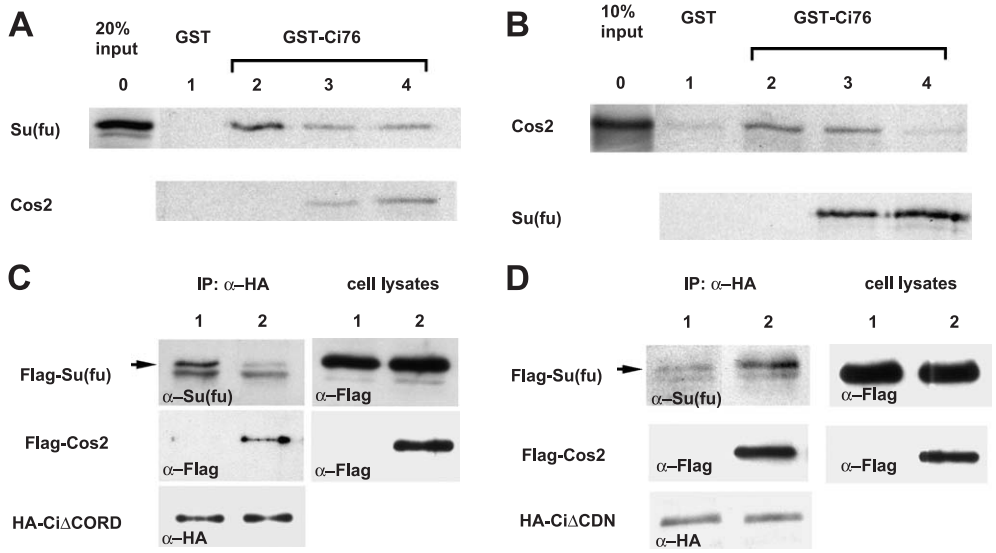

Fig. 6. Cos2 and Su(fu) compete for binding to the N-terminal region of Ci. (A) ^{35}S-labeled Su(fu) was incubated with GST (lane 1) or GST-Ci76 (lanes 2–4) in the absence (lane 2) or presence of increasing amounts of ^{35}S-labeled Cos2 (lane3, 1×; lane 4, 6×). Addition of Cos2 decreases the amount of Su(fu) pulled down by GST-Ci76. Cos2 pulled down by GST-Ci76 is shown in the bottom panel. (B) ^{35}S-labeled Cos2 was incubated with GST (lane 1) or GST-Ci76 (lanes 2–4) without (lane 2) or with increasing amounts of ^{35}S-labeled Su(fu) (lane 3, 1×; lane 4, 6×). Increasing the amount of Su(fu) reduces the amount of Cos2 pulled down by GST-Ci76. Bound Su(fu) is shown in the bottom panel. (C) S2 cells were transfected with HA-CiΔCORD and Flag-Su(fu) in the absence (lane 1) or presence of Flag-Cos2 (lane 2). Cell lysates corresponding to 20% of the materials used for IP were immunoprecipitated (IP) with a mouse anti-HA antibody, followed by immunoblotting with anti-Su(fu) (top), anti-Flag (middle) or anti-HA (bottom) antibody. Cell lysates were also immunoblotted with the anti-Flag antibody (right). Coexpression of Flag-Cos2 dramatically reduces the amount of Su(fu) bound to CiΔCORD (compare lane 1 with lane 2). Of note, the bottom band recognized the anti-Su(fu) antibody is nonspecific. (D) S2 cells were transfected with HA-CiΔCDN and Flag-Su(fu) in the absence (lane 1) or presence of Flag-Cos2 (lane 2). Cell lysates were immunoprecipitated with a mouse anti-HA antibody, followed by immunoblotting for Su(fu) (top), Flag (middle) and HA (bottom). Cell lysates corresponding to 20% of the materials used for IP were also immunoblotted with the anti-Flag antibody (right). Coexpression of Flag-Cos2 increases the amount of Su(fu) bound to CiΔCDN.

in *slimb* mutant cells remains in the cytoplasm after LMB treatment (Figs. 7A–A″). By contrast, a significant portion of Ci155 enters nucleus after treating imaginal discs with both LMB and Nocodazole (Figs. 7B–B″, C–C″). These observations suggest that an intact microtubule network is essential for cytoplasmic tethering of Ci155. Of note, nuclear translocation of Ci155 in *slimb*$^{-}$ cells situated distant from the A/P compartment boundary is not as complete as that in *slimb*$^{-}$ cells near the A/P boundary or in *cos2* mutant cells situated in similar locations (Figs. 7B,C). The residual cytoplasmic retention of Ci55 implies that a microtubule-independent mechanism may exist to impede Ci nuclear translocation (see below).

We also examined the effect of Nocodazole treatment on the subcellular localization of Ci in wild-type wing discs. Wild-type wing discs were treated with 20 ng/ml LMB with or without 40 μg/ml Nocodazole, followed by immunostaining with anti-Ci (2A1) and anti-Arm antibodies. In the absence of Nocodazole, Ci155 was accumulated in the nucleus of cells near the A/P compartment boundary (Figs. 7D,D′), whereas little if any nuclear Ci was observed in cells distant from the A/P boundary (Fig. 7D″). Upon Nocodazole treatment, A-compartment cells distant from the A/P boundary exhibited increasing levels of nuclear Ci staining (Fig. 7E″), suggesting that Nocodazole treatment promotes nuclear translocation of Ci155.

Cytoplasmic retention of Ci may involve masking of nuclear localization signal

The nuclear localization signal (NLS) of Ci is located C-terminal to the Zn finger DNA binding domain (Wang and Holmgren, 1999), and is flanked by Su(fu), Cos2, and Fu binding domains. This raises the possibility that the NLS of Ci could be masked when Ci forms complexes with Cos2, Fu, and Su(fu). NLS masking has been shown to regulate the subcellular localization of several transcription factors including NF-κB, NF-AT, and a mammalian Period protein (Beg et al., 1992; Vielhaber et al., 2000; Zhu et al., 1998). One way to test NLS masking is to add an NLS to a more exposed region of a protein and examine its effect on the subcellular localization of that protein (Beg et al., 1992; Vielhaber et al., 2000). Accordingly, an SV40 large T-antigen NLS was added to either the N-terminus of or the C-terminus of Ci as both ends of Ci are likely to be exposed in the Ci complexes (Fig. 1). *UAS-HA-Ci*$^{NLS\text{-}N}$, *UAS-HA-Ci*$^{NLS\text{-}C}$ and *UAS-HA-Ci* were expressed in wing discs using the *MS1096* Gal4 driver line. Wing discs expressing various Ci transgenes were treated with 50 ng/ml LMB, followed by immunostaining with both anti-HA and anti-Arm antibodies. As shown in Fig. 8, HA-Ci is localized mainly in the cytoplasm in A-compartment cells but translocates into the nucleus in P-compartment cells (Figs. 8A,D–E). By con-

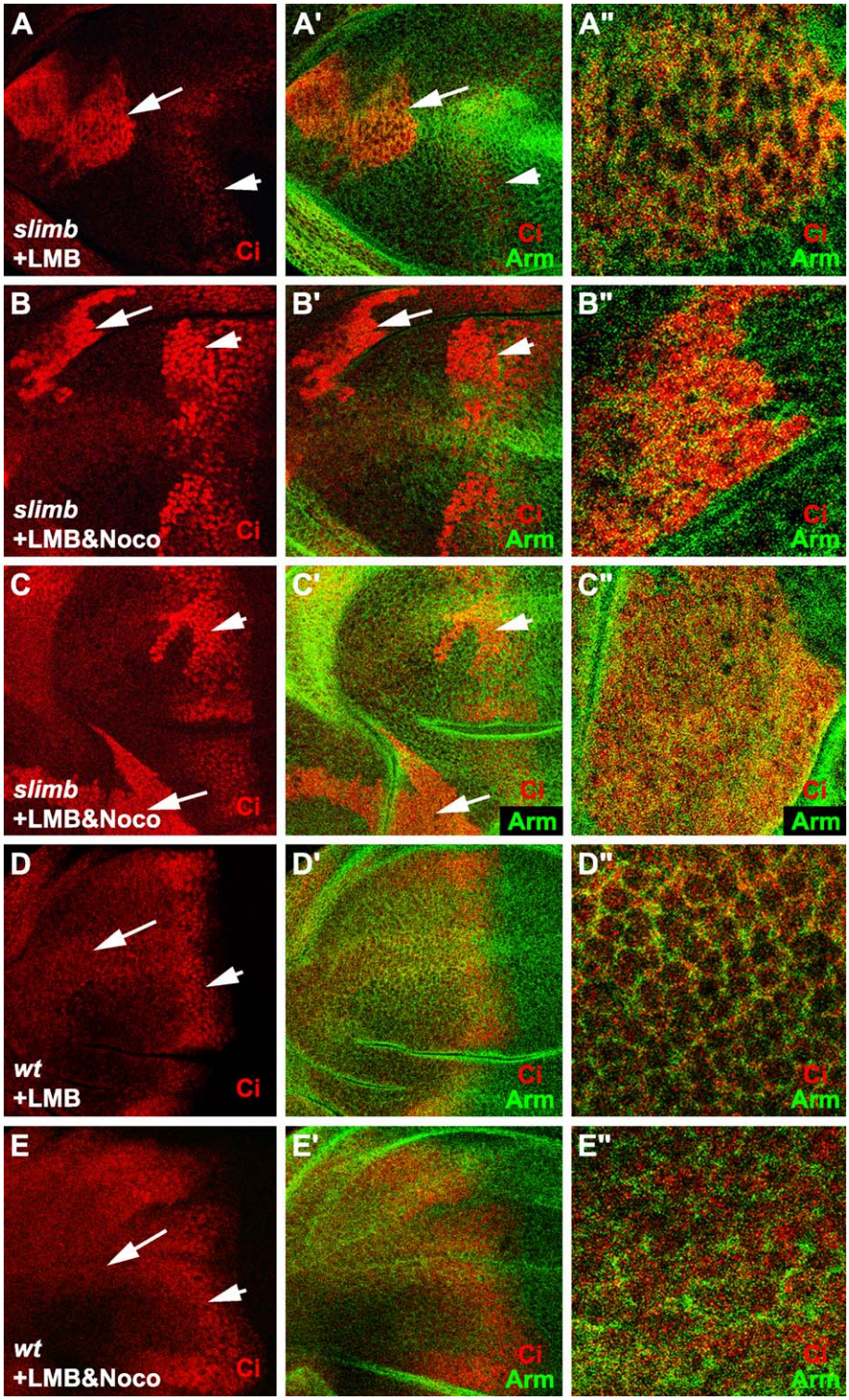

Fig. 7. The effect of Nocodazole treatment on Ci subcellular localization. Wing discs carrying *slimb*$^{-}$ clones (A–C) or wild-type wing discs (D–E) were treated with LMB in the absence (A–A″, D–D″) or presence (B–B″, C–C″, E–E″) of Nocodazole, followed by immunostaining with both anti-Ci (red) and anti-Arm (green) antibodies. Arm exhibits membrane and cytoplasmic staining. Large magnification views of the *slimb*$^{-}$ clones (indicated by the arrows in A, B and C) are shown in A″, B″ and C″. Large magnification views of wild type A-compartment cells (indicated by the arrows in D and E) are shown in D″ and E″. In the absence of Nocodazole, full-length Ci is predominantly in the cytoplasm in *slimb*$^{-}$ cells situated away from the A/P compartment boundary (A″) whereas Ci is accumulated in the nucleus in cells near the A/P boundary (arrowheads in A and A′). Nocodazole treatment induces Ci nuclear translocation (B″, C″). However, Ci nuclear translocation appears to be less complete in *slimb* mutant cells distant from the A/P compartment boundary (arrows) as compared with those near the A/P compartment boundary (arrowheads in B′ and C′). Nocodazole treatment appears to induce partial nuclear translocation of full-length Ci in wild-type A-compartment cells away from the A/P boundary (compare E″ with D″).

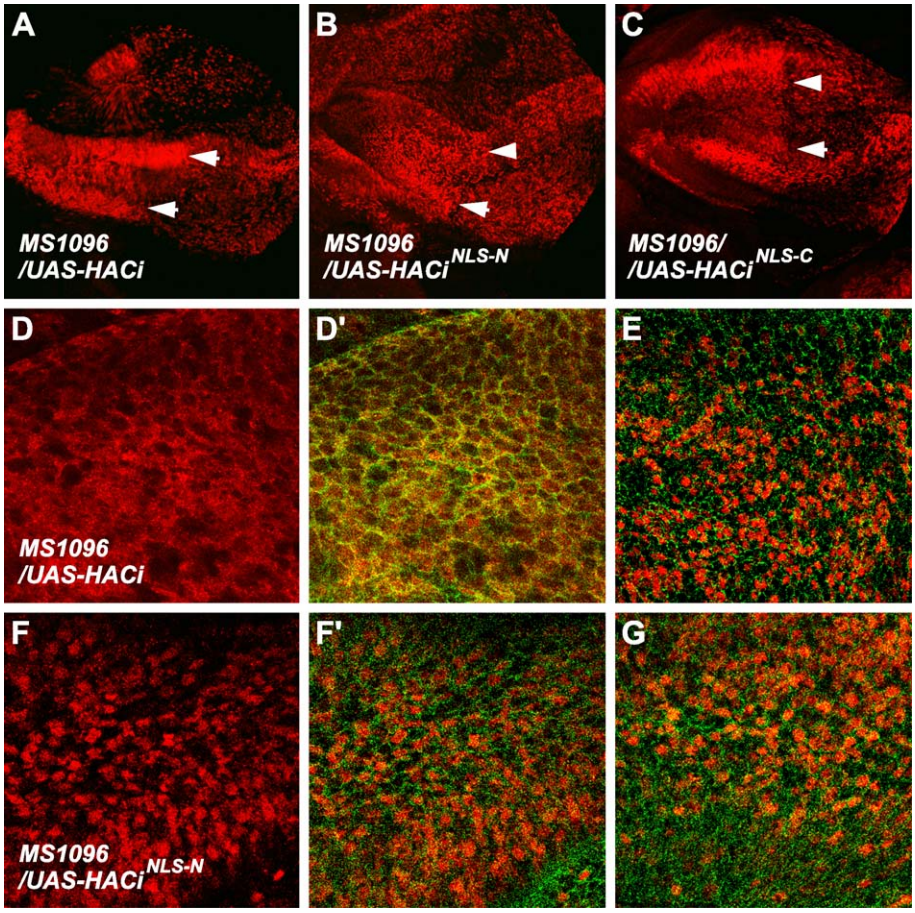

Fig. 8. The effect of adding an NLS on Ci subcellular localization. (A–C) Wing discs expressing HA-Ci (A), HA-Ci^{NLS-N} (B), and HA-Ci^{NLS-C} (C) by *MS1096* were immunostained with anti-Ci (2A) (red) and anti-Arm antibodies (green) after LMB treatment. Arrowheads demarcate the A/P compartment boundary in each panel. (D, D′ , E) Large magnification views of the A- (D, D′) and P-compartment (E) of the wing disc shown in A. (F, F′ , G) Large magnification view of the A- (F, F′) and P-compartment (G) of the wing disc shown in B. A significant amount of HA-Ci is retained in the cytoplasm in A-compartment cells (D, D′) whereas HA-Ci is located predominantly in the nucleus of P-compartment cells (E). By contrast, both HA-Ci^{NLS-N} and HA-Ci^{NLS-C} are located mainly in the nucleus both A- and P-compartment cells (C, F–G).

trast, both HA-Ci^{NLS-N} and HA-Ci^{NLS-C} are localized predominantly in the nucleus in both A- and P-compartment cells (Figs. 8B–C,F–G). Thus, adding an SV40 NLS to an exposed region of Ci has profound effect on Ci nuclear translocation, suggesting that the endogenous Ci NLS is masked.

Discussion

Control of Ci subcellular localization is an important regulatory event in Hh signal transduction. Previous studies have suggested that Ci is sequestered in the cytoplasm by forming protein complexes with Cos2, Fu and Su(fu) (for review, see Ingham and McMahon, 2001). How complex formation regulates Ci subcellular localization is not known. In this study, we provide evidence suggesting that at least two distinct mechanisms, one depends on the intact microtubule network and the other on the position of NLS within Ci, are responsible for cytoplasmic retention of Ci. We also show that Cos2 interacts with two distinct domains of Ci, CORD and CDN, both of which are involved in mediating cytoplasmic retention of Ci.

Cos2 interacts with two distinct domains of Ci to impede its nuclear translocation

We have previously identified CORD as a critical domain that mediates cytoplasmic retention of Ci deletion mutants (Wang et al., 2000). However, both CiΔCORD and Ci76 are still retained by Cos2 in our coexpression assay, implying the existence of a second Cos2 responsive element in the N-terminal region of Ci. Using yeast two-hybrid and coimmunoprecipitation assays, we found that the N-terminal region of Ci also binds Cos2, albeit with an affinity much lower than that of CORD, which may explain why it was missed by our previous study. Interaction between Cos2 and the N-terminal region of Ci has also been observed by a recent study (Monnier et al., 2002). The Ci N-terminal sequence required for Cos2 binding appears to be dispersed; never-

theless, deletion of the region from aa 346 to aa 440 compromises Cos2 binding and affects Cos2-mediated cytoplasmic retention of Ci76. Of note, under physiological condition, Ci75 is not retained effectively by Cos2 in the cytoplasm, suggesting that interaction between Cos2 and Ci75 might be intrinsically weak so that they could not form stable complexes at physiological concentrations. In addition, Ci75 lacks the NES signals in the C-terminal half of Ci (Aza-Blanc et al., 1997; Chen et al., 1999a), which allows Ci75 to be accumulated in the nucleus to repress Hh-responsive genes such as *dpp*.

Deletion of both CDN and CORD from full-length Ci renders it resistant to Cos2-mediated cytoplasmic retention whereas deletion of either CDN or CORD does not, suggesting that both domains can mediate cytoplasmic retention by overexpressed Cos2. When expressed alone in wing discs in which nuclear export is blocked by LMB treatment, HA-Ci is tethered efficiently in the cytoplasm in A-compartment cells. By contrast, HA-CiΔCORD and HA-CiΔCDN are only partially retained with HA-CiΔCDN exhibiting more nuclear staining, whereas HA-CiΔNΔC is localized near completely in the nucleus of A-compartment cells. Hence, both Cos2 binding domains are required for efficient cytoplasmic sequestration of Ci by endogenous Cos2, and CDN appears to be more potent than CORD.

Both CDN and CORD bind the N-terminal region of Cos2, but with distinct sequence requirements. The Cos2 sequence responsible for binding CORD has been narrowed down to two discrete subdomains, each of which is sufficient to bind CORD. This implies that Cos2 may bind CORD through two distinct high-affinity contacts. Alternatively, the two Cos2 subdomains may form similar binding pockets that interact with the same binding motif in CORD. By contrast, the interaction between Cos2 and CDN is mediated by large stretches of sequence from both proteins, suggesting that this interaction may involve multiple low-affinity contacts, each of which is not sufficient to mediate binding on its own. Fu binds directly to the central region of Cos2 (aa-547 to aa 750), which was also observed by Monnier et al. (2002). In addition, they further defined a Cos2 domain from aa 348 to aa 546 that binds Ci N-terminal region (Monnier et al., 2002). Hence, the Fu binding domain does not overlap significantly with Ci-binding domains in Cos2, which allows Ci and Fu to interact simultaneously with Cos2 to form large protein complexes (Robbins et al., 1997; Sisson et al., 1997; Stegman et al., 2000).

Implications on formation of distinct Cos2/Ci complexes

A previous study suggest that Cos2 and Ci can form two distinct complexes: a trimeric complex containing Cos2, Ci and Fu, and a tetrameric complex containing Cos2, Ci, Fu and Su(fu) (Stegman et al., 2000). In light of our findings that Cos2 can bind both the N-terminal and C-terminal regions of Ci, and that Su(fu) and Cos2 compete for binding to the N-terminal but not the C-terminal region of Ci, we propose that Ci and Cos2 form two distinct complexes depending on how they contact (Fig. 9). When Cos2 binds the C-terminal region of Ci, it permits Su(fu) to bind the N-terminal region. In addition, Fu interacts with both Cos2 and Su(fu) to form a tetrameric complex. However, when Cos2 binds the N-terminal region of Ci, it prevents Su(fu) from binding Ci. Fu may interact both with Cos2 and with the C-terminal region of Ci to form a trimeric complex. In support of this notion, we found that Fu can directly interact with the C-terminal region of Ci in yeast, suggesting that when Cos2 binds the N-terminal region of Ci, Fu may simultaneously interact with both Cos2 and Ci to stabilize the trimeric complex. The stoichiometric relationship between Ci and Cos2 in various Ci complexes has not been determined; therefore, we cannot exclude the possibility that one molecule of Ci may interact with a Cos2 dimer. In such a complex, Ci interacts with one molecule of Cos2 through CORD and the other through CDN.

Previous studies have shown that Ci, Cos2 and Fu are coeluted from gel filtration columns in multiple fractions that correspond to a broad range of molecular weights (Robbins et al., 1997; Sisson et al., 1997). The heterogeneity of the elution profiles implies the existence of multiple Ci complexes in vivo. Why Ci forms multiple distinct complexes is not clear; however, different complexes may possibly play different roles in regulating Ci processing, subcellular localization, or activity. In addition, different complexes may respond to distinct thresholds of Hh signaling activities. For example, only high levels of Hh activate full-length Ci by inhibiting Su(fu) (Ohlmeyer and Kalderon, 1998). Thus, Su(fu) containing Ci complexes might only respond to high doses of the Hh signal.

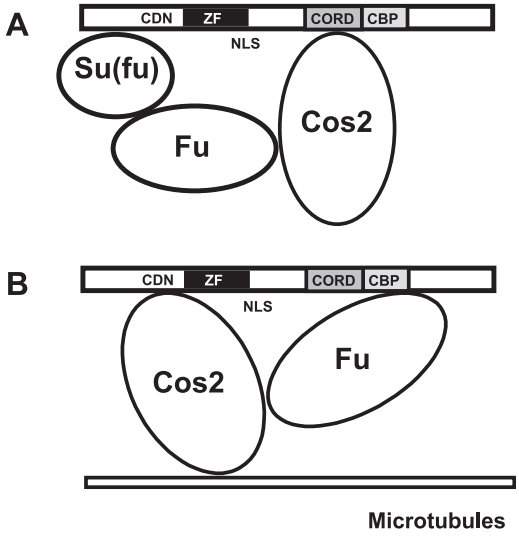

Fig. 9. Proposed model for distinct Cos2/Ci complexes. (A) A tetrameric complex forms when Cos2 binds CORD, allowing Su(fu) to bind the N-terminal region of Ci. Fu connects Cos2 to Su(fu). (B) A trimeric complex forms when Cos2 binds CDN, preventing Su(fu) from binding to Ci. Fu may contact both Cos2 and the C-terminal region of Ci. See text for detail.

Microtubule-dependent and -independent mechanisms for regulating Ci subcellular localization

Based on the observation that Cos2, Fu and Ci cosediment with microtubules in a manner regulated by Hh, it has been proposed that Cos2 tethers Ci by binding to microtubules, and Hh induces Ci nuclear translocation by dissociating Cos2/Fu/Ci complex from microtubules (Ingham and McMahon, 2001). Furthermore, it has been speculated that Cos2 might regulate Ci processing by tethering it to microtubules (Kalderon, 1997; Robbins et al., 1984; Sisson et al., 1997). We show here that Nocodazole treatment facilitates Ci nuclear translocation but does not induce the accumulation of full-length Ci. Hence, it appears that an intact microtubule network is required for efficient cytoplasmic retention of Ci but is not essential for proteolytic processing of Ci.

Although Nocodazole treatment causes Ci nuclear import, the effect is not as dramatic as that caused by removal of Cos2 function, implying that Cos2 may use a microtubule-independent mechanism to sequester Ci. Indeed, we found that cytoplasmic retention of Ci also relies on the position of NLS. Ci has a bipartite NLS located at the C-terminal end of the Zn-finger DNA binding domain (Wang and Holmgren, 1999), which is flanked by Su(fu), Cos2 and Fu interaction domains. Addition of an NLS to either the N- or C-terminus of Ci greatly facilitates its nuclear translocation, suggesting that the NLS of Ci is masked by complex formation.

It has been shown previously that Su(fu) impedes Ci nuclear translocation by direct binding (Methot and Basler, 2000; Wang et al., 2000). As Su(fu) does not cosediment with microtubules (Stegman et al., 2000), Su(fu) must use a microtubule-independent mechanism to regulate Ci subcellular localization. One likely mechanism by which Su(fu) impede Ci nuclear import is to form a tetrameric complex with Cos2, Fu and Ci to mask Ci NLS (Fig. 9).

How does Hh signaling stimulate Ci nuclear translocation? It has been shown that Hh induces dissociation of Ci/Cos2/Fu complexes from microtubules (Robbins et al., 1997), suggesting that Hh may promote Ci nuclear import by releasing it from microtubule tethering. However, our finding that NLS masking also plays an important role in cytoplasmic retention of Ci implies that Hh signaling may further induce disassembly or conformational change of Ci complexes to expose its NLS. Consistent with this, it has been recently shown that Hh signaling appears to dissociate Ci from Cos2/Fu complex (Ruel et al., 2003).

Acknowledgments

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